

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/05717

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522340 A	24-08-1995	AU 695111 B	06-08-1998
		AU 1878795 A	04-09-1995
		AU 5942298 A	04-06-1998
		CA 2183564 A	24-08-1995
		CA 2219948 A	24-08-1995
		EP 0751782 A	08-01-1997
		EP 0839831 A	06-05-1998
		JP 9509418 T	22-09-1997

EP 0785215 A	23-07-1997	AU 7037896 A	01-05-1997
		BR 9605220 A	21-07-1998
		CA 2188508 A	25-04-1997
		JP 9176039 A	08-07-1997
		US 5691455 A	25-11-1997

INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SLADE M B ET AL.: "Expression of recombinant glycoproteins in the simple eukaryote Dictyostelium discoideum" BIOTECHNOLOGY AND GENETIC ENGINEERING REVIEWS, vol. 14, 14 April 1997, pages 1-35, XP002055617 cited in the application see page 6, paragraph 4 - page 14, paragraph 2 see page 16, paragraph 4 see page 23, paragraph 3 ----	1-7
A	WO 95 22340 A (SENSI TEST) 24 August 1995 see abstract; figures 6-15, 18; examples 6, 11-21; table 1 see page 1, paragraph 1 see page 10, line 33 - page 13, line 27 ----	1-7
A	EP 0 785 215 A (AKZO NOBEL NV) 23 July 1997 see abstract see the claims see page 2, line 49 - page 5, line 34; figures 3-9; examples 1-3, 7-12 ----	1-6
P, X	HEIKOOP J C ET AL.: "Expression of a bioactive, single-chain choriogonadotropin in Dictyostelium discoideum" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 256, no. 2, 1 September 1998, pages 359-363, XP002089111 see the whole document -----	1-7

INTERNATIONAL SEARCH REPORT

Inter. Appl. No.
PCT/EP 98/05717

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/16 C12N15/80 C07K14/59 A61K38/24 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>HEIKOOP JC ET AL.: "Evaluation of subunit truncation and the nature of spacer for single chain human gonadotropins" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 245, no. 3, May 1997, pages 656-662, XP002055951 cited in the application see the whole document</p> <p style="text-align: center;">--- -/--</p>	1-7



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search

5 January 1999

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Name and mailing address of the ISA

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Authorized officer

Oderwald, H

10/10

(ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCAGATCTAA ACATTTAAGA TTTGTG

26

(2) INFORMATION FOR SEQ ID NO: 5:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTATCGACA TATGCAAATG CATCCAAGGA GCCGCTTCG

39

9/10

Met Lys Phe Gln His Thr Phe Ile Ala Leu Leu Ser Leu Leu Thr Tyr

1

5

10

15

Ala Asn Ala

5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 74 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

20 GCAGATCTAT GGAGATGTTC CAAGGTCTCC TCCTTTTATT ACTCCTCAGC ATGGGTGGTA 60

CATGGGCATC CAAG 74

(2) INFORMATION FOR SEQ ID NO: 4:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

30 (D) TOPOLOGY: linear

8/10

(ii) MOLECULE TYPE: cDNA

5 (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 6..62

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGATC ATG AAA TTC CAA CAT ACA TTT ATT GCA TTA TTA TCA CTA TTA 47

Met Lys Phe Gln His Thr Phe Ile Ala Leu Leu Ser Leu Leu

1

5

10

15

ACA TAT GCA AAT GCA GATCT

67

Thr Tyr Ala Asn Ala

15

20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

25 (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

30

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

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10 (F) POSTAL CODE (ZIP): 6824 BM

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(H) TELEFAX: 0412 650592

(ii) TITLE OF INVENTION: Expression of gonadotropins in Dictyostelium

15

(iii) NUMBER OF SEQUENCES: 5

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20 (B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 67 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

30 (D) TOPOLOGY: linear

6/10

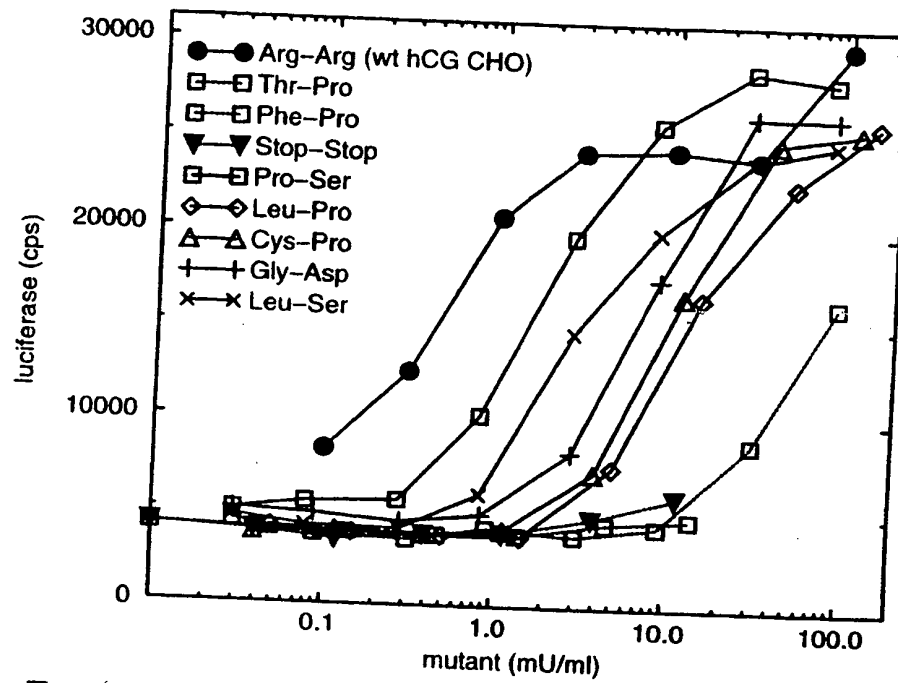


Fig. 6



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: C12N 15/16, 15/80, C07K 14/59, A61K 38/24, G01N 33/50	A1	(11) International Publication Number: WO 99/13081 (43) International Publication Date: 18 March 1999 (18.03.99)
(21) International Application Number: PCT/EP98/05717 (22) International Filing Date: 2 September 1998 (02.09.98) (30) Priority Data: 97202757.7 8 September 1997 (08.09.97) EP (71) Applicant (for all designated States except US): AKZO NOBEL N.V. [NL/NL]; Velperweg 76, NL-6824 BM Arnhem (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): GROOTENHUIS, Peter, Diederik, Jan [NL/NL]; Van der Duyn van Maasdamstraat 48, NL-5344 HS Oss (NL). HEIKOOP, Judith, Christina [NL/NL]; Zusters van Orthenpoort 8, NL-5211 ND s'Hertogenbosch (NL). LINSKENS, Maarten, Herman, Karel [NL/NL]; Handellaan 20, NL-9722 JX Groningen (NL). VAN HAASTERT, Petrus, Johannes, Maria [NL/NL]; Wolddijk 137, NL-9738 AE Groningen (NL). BLAAUW, Mieke [NL/NL]; Briljantstraat 131, NL-9743 NE Groningen (NL). (74) Agent: KRAAK, H.; P.O. Box 20, NL-5340 BH Oss (NL).		(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, ID, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: EXPRESSION OF GONADOTROPINS IN <i>DICTYOSTELIUM</i>		
(57) Abstract <p>The present invention relates to gonadotropins expressed in <i>Dictyostelium</i>. The gonadotropins are found to be secreted in a biological active form. Expression of gonadotropins in <i>Dictyostelium</i> provides an easy way to select for gonadotropin mutants.</p>		

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EXPRESSION OF GONADOTROPINS IN DICTYOSTELIUM

The invention relates to gonadotropins or mutants thereof expressed in *Dictyostelium*, pharmaceutical compositions containing the same, a method for the preparation of the gonadotropins as well as a method for the selection of gonadotropin mutants with superagonistic or antagonistic properties.

The gonadotropins form a family of structurally related glycoprotein hormones. Typical members include chorionic gonadotropin (CG), follicle stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). FSH, LH and TSH are present in most vertebrate species and are synthesized and secreted by the pituitary. CG has so far been found only in primates, including humans, and in horses and is synthesized by placental tissue.

The hormones are heterodimeric proteins of around 30 kD formed by a non-covalent association of a common α -subunit and a hormone specific β -subunit.

Within a species, the α -subunit is essentially identical for each member of the gonadotropin family; it is also highly conserved from species to species. The β -subunits are different for each member, i.e. CG, FSH, TSH and LH, but show considerable homology in structure. Furthermore, also the β subunits are highly conserved from species to species. In humans, the α subunit consists of 92 amino acid residues, whilst the β subunit varies in size for each member: 111 residues in hFSH, 121 residues in hLH, 118 residues in hTSH and 145 residues in hCG (Combarnous, Y. (1992), Endocrine Reviews, 13, 670-691, Lustbader, J.W. et al. (1993), Endocrine Reviews, 14, 291-311). The β subunit of hCG is substantially larger than the other β subunits in that it contains approximately 34 additional amino acids at the C-terminus referred to herein as the carboxy terminal peptide (CTP). This CTP bears four serine linked oligosaccharides.

The gonadotropins serve important functions in a variety of bodily functions including metabolism, temperature regulation and the reproductive process. The hypophyseal gonadotropin FSH for example plays a pivotal role in the stimulation of follicle development and maturation whereas LH induces ovulation (Sharp, R.M. (1990), Clin Endocrinol., 33, 787-807; Dorrington and Armstrong (1979), Recent Prog. Horm. Res., 35, 301-342). Currently, FSH is applied clinically, either alone or in combination with LH, for ovarian stimulation i.e. ovarian hyperstimulation for *in*

vitro fertilisation (IVF) and induction of *in vivo* ovulation in infertile anovulatory women (Inslar, V.(1988), Int. J. Fertility, 33, 85-97, Navot and Rosenwaks (1988), J. Vitro Fert. Embryo Transfer, 5, 3-13), as well as for male hypogonadism. The human choriogonadotropin (hCG) is involved in the maintenance of pregnancy in the early stages after conception and has also important therapeutic applications.

The two subunits of the heterodimer display many conserved intra-subunit disulfide bonds: five disulfide bridges in the α -subunit and six disulfide bridges in the β -subunit. The corresponding cysteine residues are fully conserved among all members of the gonadotropin family. The recently obtained X-ray structure of hCG shows that these disulfide bonds are involved in typical three-dimensional patterns called disulfide knots.

The gonadotropins possess three or four asparagine residues that can be N-glycosylated and have an important impact on its conformation and biological activity. In addition the C-terminal peptide (CTP) of hCG can be O-glycosylated at four serine positions. The major role of the glycosylated CTP seems to be the prolongation of the circulatory half-life of hCG.

The biosynthesis of the glycoprotein hormones is a highly complex process. In the last decade it has become clear that folding, assembly and secretion of gonadotropins is assisted by a large set of chaperones and folding enzymes, residing in the Endoplasmic reticulum and the Golgi apparatus. Since both the α - and the β -subunit contain a so-called cystine-knot, it can be anticipated that protein disulfide isomerase plays a key role in the facilitation of the folding process. In addition, it has been shown that the N-linked oligosaccharide side chains are required for proper folding, disulfide formation and secretion of hCG (Feng, W. et al (1995) J. Biol. Chem. 270, 11851-11859). The successful assembly of the two subunits into the dimer is an absolute prerequisite for the biological activity of the dimer.

Elucidation of functional determinants of the heterodimeric glycoprotein hormones is often hampered by unwanted secondary effects of mutations on the assembly of the subunits. To facilitate structure/function analyses studies mutants have been produced in which the coding regions of the hCG β -subunit and the common α -subunit are

connected via peptide spacers or intersubunit disulfide bonds. It has been shown that these covalently linked α - and β -subunits of hCG are able to fold into biologically active conformations.

5 The gonadotropins have been expressed in Chinese Hamster Ovary (CHO) cells, and their recombinant derivatives have biological activities comparable to the native hormones (Olijve, W. et al. (1996) Mol. Hum. Reprod. 2, 371-382). This indicates that these host cells contain all chaperones and folding enzymes necessary to assemble the α - and β -subunits of hCG and to perform all post-translational modifications necessary for full biological activity. Furthermore, the use of CHO cells
10 in combination with site-directed mutagenesis has proven to be a valuable tool for elucidating functional determinants in the glycoprotein hormones (Puett, D. et al H. (1994) in *Glycoprotein Hormones* (Lustbader, J.W., Puett, D. & Ruddon, R.W., Eds.) pp. 59-82, Springer-Verlag, New-York) and for designing potential new therapeutic analogs of these hormones (Fares, F.A. et al. (1992), Proc Natl Acad Sci U S A 89, 4304-4308).
15

Unfortunately, the use of mammalian cells such as CHO cells for the expression of human gonadotropins suffers from some limitations. Thus, it is not possible to generate the high number of transformants necessary for studies that involve random mutagenesis
20 of protein domains. Random mutagenesis of selected domains in proteins has been shown to be a valuable tool for identifying the structural determinants for receptor binding and bioactivity. In addition, the use of CHO cells is expensive and labour intensive. Thus, there is a need for the expression of gonadotropins in a more robust expression host.

25 Host cells derived from lower organisms might meet the above mentioned requirements, but it is expected that the complex folding of the gonadotropins hampers proper expression and secretion of such complex recombinant proteins.

Now, unexpectedly it has been found that *Dictyostelium* is capable of producing the highly complex glycoprotein hormones.

30

The soil amoebae *Dictyostelium discoideum* is an organism that provides an attractive alternative for heterologous expression of the human glycoprotein

hormones. While it can be grown and transformed with the same ease as the yeast *Saccharomyces*, it has some of the complex features that resemble mammalian cells, such as glycosylation and chemotaxis. Furthermore, it has recently been shown that *Dictyostelium* provides a useful system for random mutagenesis approaches.

5 Nevertheless, there have been found differences between the glycosylation of proteins produced in *Dictyostelium* compared to material produced in CHO cells. It is known that glycosylation plays an important role in hormone function, and whereas most glycosylation is performed by *Dictyostelium*, galactose, N-acetylgalactosamine or sialic acids are not attached to the oligosaccharide side chains (Slade, M. et al. (1997) 10 Biotech. Genet. Eng. Rev., 14, 1-35). Though the post-translational modification is not identical to that in higher eukaryotes, gonadotropins produced in *Dictyostelium* also were found to be biologically active. The protein is found to be capable of binding to its receptor and to stimulate cAMP production in cells expressing the human LH/CG receptor.

15 The heterogeneity of the expressed glycoproteins is greatly reduced leading to much more homogeneous preparations of isohormones. Therefore, gonadotropins produced in *Dictyostelium* are chemically well-defined relative to the more complex CHO-produced gonadotropins. This is a major advantage for the maintenance and analytical validation of the batch-to-batch consistency. Since the glycosylation is a 20 very important determinant for the *in vivo* half-live of gonadotropins, production of gonadotropins in *Dictyostelium* provides a tool to produce non-mutated gonadotropins with well-defined *in vivo* half-lives. In addition, combination of the expression in *Dictyostelium* with protein engineering facilitates tailor made gonadotropins for several clinical applications.

25 Because of the complex inter and intra molecular folding of the two subunits, the large number of disulfide bridges, disulfide knots and post-translational modifications, it is remarkable that active gonadotropins can be expressed in *Dictyostelium* and that properly folded molecules can be prepared according to the invention. Proper disulfide bond formation is a critical event in the folding and 30 maturation of functional gonadotropins. Especially the disulfide bond formation in the β subunit is critical: all disulfide bonds are required for efficient combination and folding. Detailed studies of the folding of hCG revealed that the folding of the molecule does not proceed by a simple sequential pathway, but proceeds

independently in different domains of the molecule. It was therefore thought that cells of lower organisms were not capable of secreting properly folded gonadotropins.

The present invention provides for gonadotropins expressed in *Dictyostelium*.

5 Use of *Dictyostelium discoideum* has the advantage that is a well-studied organism. The vegetative amoebae are easy and inexpensive to grow either in axenic culture or on Gram-negative bacteria. Furthermore, several transformation vectors have been described which are capable of directing expression of foreign proteins.

The gonadotropins according to the invention can be dimeric i.e. composed of two non-covalently bound subunits. Preferably, the gonadotropin is hCG or FSH. The
10 gonadotropins, however, can comprise modifications generally known in the art.

In one such preferred modification of the gonadotropins according to the invention, the C-terminus of the amino acid sequence of one of the subunits is linked, optionally through a linker moiety, to the N-terminus of the amino acid sequence of the other subunit. Preferably the linker moiety is a complete or partial CTP unit or
15 variant thereof, or a repeated oligopeptide e.g. a 5 times repeated Ser-Gly peptide.

Another modification of the gonadotropins according to the invention can be an extension of the α and/or β subunit at their respective N- or C-terminus with a complete or partial CTP unit or a variant thereof. The extension may comprise the respective CTP units in single or multiple forms. Alternatively, a complete CTP unit
20 or partial CTP unit or multiple forms thereof can be inserted in the N- or C-terminus of said subunits. Again another modification is the introduction of one or more non-native disulfide bridges.

Furthermore, the gonadotropins according to the invention may be either glycosylated or partially glycosylated. Partially glycosylated gonadotropins according
25 to the invention can be obtained by site-directed mutagenesis whereby one or more of the glycosylation recognition sites in the gonadotropins are removed. Alternatively, the glycosylation pattern of the gonadotropins according to the invention can be modified by the introduction of additional glycosylation recognition sites and, optionally, the removal of one or more glycosylation recognition sites, resulting in a
30 modified glycosylation of said gonadotropins. A glycosylation recognition site as used herein consists of the amino acid sequence Asn-X-Ser/Thr, wherein X can be any amino acid.

As used herein, the α and β subunits of CG, FSH, LH and TSH as well as the heterodimeric forms have in general their conventional definitions and refer to the proteins having the amino acid sequences known in the art *per se*, or allelic variants thereof, regardless of the glycosylation pattern displayed.

5 “Native” forms of these proteins are those proteins which have the amino acid sequences as isolated from the relevant vertebrate tissue, and have these known sequences *per se*, or their allelic variants thereof.

 These “variants” are those proteins which have deliberate alterations in amino acid sequences relative to the native proteins. The alterations may include single or
10 multiple deletions, insertions, substitutions and combinations thereof, and can be produced by, for example, site specific mutagenesis.

 As used herein, the “CTP unit” refers to the amino acid sequence found at the carboxy terminus of the β subunit of hCG which extends from amino acid 112-118 to residue 145 at the C-terminus or to a portion thereof. A “complete” CTP unit contains
15 28-34 amino acids, depending on the N-terminus of the CTP. A “partial” CTP unit is an amino acid sequence which occurs between positions 112-118 to 145 inclusive, but which has at least one amino acid deleted from the shortest possible complete CTP unit (amino acid 118-145). “Multiple” CTP units are understood to encompass tandem arrays of the complete CTP unit or partial CTP unit or combinations of both.

20 Methods to construct the gonadotropin genes according to the invention are well known in the art (Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, latest edition). Techniques for site directed mutagenesis, ligation of additional sequences, PCR, and construction of suitable expression systems are all, by now, well known in the art. Portions or all of
25 the DNA encoding the desired protein can be constructed synthetically using standard solid phase techniques, preferably to include restriction sites for ease of ligation. Suitable control elements for transcription and translation of the included coding sequence can be provided to the DNA coding sequences.

 The invention also provides for a method for the expression of gonadotropins or
30 mutants thereof in *Dictyostelium*. Said method according to the invention comprises the steps of:

- transforming a strain of *Dictyostelium* with a recombinant plasmid vector comprising a DNA sequence encoding the gonadotropin genes or mutated genes

under control of *Dictyostelium* regulatory sequences

- culturing the recombinant strain under conditions to allow expression of the DNA sequence and
- isolating the expressed protein.

5 Preferably the protein to be expressed is a single-chain protein, i.e. the subunits are covalently connected through a spacer molecule. More preferably the gonadotropin is single-chain hCG.

Another aspect of the invention is to provide a method to easily screen for mutated gonadotropins. Said method comprises :

- 10 - random mutagenesis of gonadotropin genes
- insertion of the mutated genes in a *Dictyostelium* plasmid vector
- transforming a strain of *Dictyostelium* with said recombinant plasmid vector
- culturing clones under conditions to allow expression of the DNA sequence
- determining the receptor binding / signal transduction ratio and
- 15 - isolating clones with a ratio deviating from the ratio determined for wild type gonadotropins.

Preferably, the mutated gonadotropins show a receptor binding which equals the binding of the native protein to its receptor. More preferably, the affinity of the mutated protein to its receptor is higher than its native counterpart. The signal
20 transduction has to be at least two-fold higher or lower as compared to the native protein. Preferably, the difference in signal transduction amounts a factor 10.

Proteins exhibiting a high ratio are useful as antagonists whereas proteins with a low ratio can be used as super agonists.

Methods to determine receptor binding as well as *in vitro* and *in vivo* assays to
25 determine biological activity of gonadotropins are well known.

Random mutagenesis need not to be performed on the complete gonadotropin gene but instead can also be carried out on a single subunit gene or a well-defined region such as e.g. the determinant loop.

30 In order to introduce random point mutations in the gonadotropin gene(s), amplification of the region(s) of interest with Taq DNA polymerase can be employed. Since Taq DNA polymerase lacks a 3'→5' exonucleolytic editing activity, this enzyme is an error-prone DNA polymerase, with a measured error rate of 10⁻⁵ to 10⁻⁴ error per nucleotide synthesized. Therefore, use of PCR with Taq DNA polymerase

under essentially standard reaction conditions can be used to introduce mutations (Zhou, Y. et al. (1991), Nucl. Acids Res. 19, 52). However, the frequency of mutations using these conditions is adequate for mutagenizing relatively large sequences, but not for small DNA fragments (< 500 bp). The infidelity of Taq DNA polymerase can be increased by addition of Mn^{2+} and the use of relatively high concentrations of dNTP and Mg^{2+} (Leungh, D.W. et al. (1989), Technique 1, 1-15). An alternative method for the adjustment of the mutation frequency, which also offers the opportunity of influencing the types of mutation, is the use of dITP in combination with limiting amounts of one of the four dNTPs (Spee, J.H. et al. (1993), Nucl. Acids Res. 21, 777-778). For mutagenesis of short target DNA (< 50 bp), the use of degenerate oligonucleotides seems to be the method of choice (Kirchhoff, F. and Desrosiers, R.C. (1996), Meth. Molec. Biol. 57, 323-333). Usually, the procedures have four steps. In step one, the region of interest is amplified by (modified) PCR. In step two, the amplified DNA is digested with a pair of restriction endonucleases that cut at each end of the DNA sequence of interest. In step three, the DNA fragment containing the DNA sequence of interest is ligated with restriction endonuclease digested vector DNA. In step four, the resulting recombinant DNA molecules are introduced into the cells by transformation or electroporation.

DNA vectors encoding any of the gonadotropins according to the invention are also within the scope of the invention. DNA vectors according to the invention can be obtained by operatively linking the DNA encoding the native gonadotropins or variants thereof to DNA comprising *Dictyostelium* regulatory sequences. Optionally, these vectors also might contain regions which contain origins of replication and/or polypeptide-encoding sequences facilitating extrachromosomal replication. Such vectors have the advantage that they can replicate extrachromosomally in the *Dictyostelium* host cell.

As explained, the variant gonadotropins according to the invention can be agonists or antagonists, depending on the mutation site. The mutation site may lead to subtle changes in the conformation of the molecule. If the mutation site e.g. is selected in parts of the protein that are associated with receptor binding and/or signal transduction, the excreted protein according to the invention may lead to a partial or complete loss of signal transduction activity. Such altered gonadotropins, wherein the

receptor binding properties are retained, can be used as antagonists. Also gonadotropins with improved binding and signal transduction activities may be selected.

5 The agonist gonadotropins according to the invention can be used for the same clinical purposes as the native gonadotropins. In addition the proteins can be used as diagnostic tools to detect the presence or absence of antibodies with respect to the native proteins in biological samples. They are also useful as control reagents in assay kits for assessing the levels of gonadotropin hormones in various samples. Antagonists can be used e.g. in the treatment of gonadotropin dependent tumors;
10 LH/hCG antagonists to prevent LH surges during controlled ovarian hyperstimulation and FSH antagonists for male contraception.

Suitable pharmaceutical compositions according to the invention comprise one or more of the gonadotropins according to the invention and a pharmaceutical acceptable carrier.

15 Pharmaceutical acceptable carriers are well known to those skilled in the art and include, for example, sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water.

Furthermore the pharmaceutical composition according to the invention may comprise one or more stabilizers such as, for example, carbohydrates including
20 sorbitol, mannitol, starch, sucrodedextrin and glucose, proteins such as albumin or casein, and buffers like alkaline phosphates.

Suitable administration routes are intramuscular injections, subcutaneous injections, intravenous injections or intraperitoneal injections, oral and intranasal administration.

25

The following examples are illustrative for the invention and should in no way be interpreted as limiting the scope of the invention.

LEGENDS TO THE FIGURES

Figure 1: Plasmids and cloning strategy. (1A) Maps of plasmids MB12n and MB12n/PsA. Both plasmids consists of four main fragments, containing *E.coli* maintenance sequences (bluescript), blasticidine resistance gene under Dd promoter control (BSR), a cloning cassette with Dd promoter (act15) and terminator (2H3), and sequences for extrachromosomal maintenance in *Dictyostelium* (G4/D5, G5/D6). MB12n/PsA contains a PsA linker sequence (Table 1) inserted into the unique BglII site of MB12n. (1B) Cloning diagram of JV158. The oligonucleotides used are shown by the horizontal arrows. The xxx shown in oligonucleotide b20aarev indicate that sequence substitutions have been made to optimize the sequence for *Dictyostelium* codon preference. (1C) Cloning diagram of JV10PSA. All oligonucleotide sequences are given in Table 1.

Figure 2: Binding activity of wildtype hLH, hCG and single chain hCG (sc hCG) produced in Chinese Hamster Ovary cells (CHO) or *Dictyostelium discoideum* (Dd) to membranes of CHO cells stably expressing the human LH/CG receptor. The membranes were incubated with ¹²⁵I-labeled hCG in the absence or presence of varying concentrations unlabeled wildtype hLH, hCG or the single chain hCG's. Displacement curves are presented as the percentage of maximal binding at each dose of unlabeled hormone.

Figure 3: *In vitro* biological activity of wildtype hLH, hCG and single chain hCG (sc hCG) produced in Chinese Hamster Ovary cells (CHO) or *Dictyostelium discoideum* (Dd). Extracellular cAMP was measured by specific RIA after stimulation of CHO cells stably expressing the human LH/CG receptor.

Figure 4: *In vitro* biological activity of wildtype hCG produced in Chinese Hamster Ovary cells (CHO) or *Dictyostelium discoideum* (Dd). Luciferase production was measured after 4 h incubation at 37 °C and stimulation of CHO cells stably expressing the human LH/CG receptor and a reporter construct.

Figure 5: *In vitro* biological activity of wildtype FSH produced in Chinese Hamster Ovary cells (CHO) or *Dictyostelium discoideum* (Dd). Luciferase production was measured after 4 h incubation at 37 °C and stimulation of CHO cells stably expressing the human FSH receptor and a reporter construct.

5

Figure 6: *In vitro* biological activity of wildtype hCG from CHO cells and selected hCG mutants produced by *Dictyostelium discoideum* (Dd). Luciferase production was measured after 4 h incubation at 37 °C and stimulation of CHO cells stably expressing the human LH/CG receptor and a reporter construct.

10

EXAMPLES

Example 1:

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Design of the expression constructs

A gonadotropin mutant consisting of a completely intact α -subunit connected via a peptide decamer containing five Ser-Gly repeats to the β -chain only lacking its C-terminal peptide, was selected for expression in *Dictyostelium*.

20

Two constructs were generated, which differ in their leader sequences. The first one contains the natural leader sequence of the β -subunit of hCG. To limit possible problems in mRNA translation due to the presence of a considerable amount (about 40% in the β -subunit of hCG) of infrequently used codons, the first 30 bases of the coding sequence were altered conform to *Dictyostelium* preferred codon usage. For the construction of the second construct it was considered that the proteins involved in the secretion route of mammalian cells, via the ER and Golgi, may not be conserved between different species. To facilitate transport in *Dictyostelium*, the leader peptide of the human β -subunit was exchanged with a leader peptide of a *Dictyostelium* glycoprotein, Prespore protein A (PsA), which is transported over the plasma membrane. This leader had been used previously to express secreted heterologous proteins (Dittrich, W. et al. (1994) Bio/Technology 12, 614-618).

30

The expression plasmids were called JV158 (adapted codons with β -subunit leader peptide) and JV10PSA (with the PsA leader peptide) and were derived from MB12n.

5 MB12n is an 8.25 kb extra-chromosomally maintained *Dictyostelium discoideum* (Dd) plasmid, containing an unique restriction site between a Dd promoter and terminator. MB12n consists of 2.9 kb (a ClaI -HincII partial digest fragment) from p155d1 (Hughes, J.E. et al. (1994) Mol. Cell. Biol. 14, 6117-6124) containing the Dd origin of replication and two Dd genes required for replication (G4/D5 and G5/D6), 2.95 kb from pBluescript (Stratagene) for propagation in *E. coli*, 1.35 kb containing
10 the blasticidine resistance gene between the Dd Actine 15 promoter and the Dd Actin 8 terminator (from pBsr2, Sutoh, K. (1993), Plasmid 30, 150-154), and a 1.05 kb cloning cassette containing the Dd Actin 15 promoter, an unique BglII restriction site and the Dd 2H3 terminator (from BS18.2H3, Kumagai A. et al (1989) Cell 57, 265-275). The BglII site in the blasticidine resistance gene has been removed by
15 mutagenesis. Figure 1A illustrates the relative position of these components in MB12n. Plasmid MB12n/PsA contains a linker sequence encoding the 19 amino acid PsA leader peptide (Early, E.A. et al (1988) Mol. Cell. Biol. 8 3458-3466; Dittrich, et al (1994) Bio/Technology 12, 614-618).cloned in the unique BglII site. The plasmid has an unique NdeI site in the 3' part of the PsA sequence and an unique BglII site 5'
20 of the 2H3 terminator, to facilitate 'in frame' directional cloning. The BglII site adjacent to the Dd Actin15 promoter was eliminated during cloning (see Table 1).

Using primers b20aarev and alphater (Table 1) a single chain hCG was amplified by PCR from a plasmid containing hCG mutant 1 [β -(1-111)-(Ser-Gly)₅- α -(1-92)], (Heikoop, J. C. et al. (1997) Eur. J. Biochem. 245, 656-662). The resulting
25 fragment was cloned in MB12n after digestion with BglII. Primer b20aarev is designed to optimize the first 10 amino acids of the β chain for codon usage in *Dictyostelium*. The same template was amplified with primers bmature (Table 1) and alphater to produce a product that, after digestion with NdeI and BglII, was cloned into MB12n/PsA. The resulting plasmids were labeled JSV158 and JV10PSA,
30 respectively (see Figure 1B and 1C). PCR was performed with the Expand High Fidelity (Boehringer Mannheim) system, using the following cycle parameters: denaturation for 3 minutes at 94 °C, then 25 cycles with 30 seconds at 94 °C, 30 seconds at 37 °C and 60 seconds at 72 °C. PCR products were separated by gel electrophoresis, excised and purified with Qiaex II (Qiagen) before restriction

digestion and cloning. All DNA sequences were analyzed and confirmed by dideoxy sequencing.

Table 1: DNA sequences for oligonucleotides and PsA linker. The PsA sequence also shows the peptide sequence of the PsA leader, as well as the unique restriction sites BglII and NdeI.

Oligo ID: Sequence:

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10   PsA:      (BglII)                                NdeI      BglII
      agatcatgaa attccaacat acatttattg cattattatc actattaaca tatgcaaatg cagatct
      M K F Q H T F I A L L S L L T Y A N A
      b20aarev: gcagatctat ggagatgttc caaggtctcc tccttttatt actcctcagc atgggtggta
      catgggcac caag
15   alphater: ccagatctaa acatttaaga ttgtg
      bature:  gctatcgaca tatgcaaatg catccaagga gccgcttcg
-----

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20 **Example 2:**

Expression of single chain hCG in Dictyostelium

Dictyostelium strain AX3 was grown to a density of 2×10^6 cells per ml in axenic medium before electroporation. The electroporation conditions were basically as described (Mann S.K.O. et al (1994) in: Cell Biology: a Laboratory Handbook, J.E. Celis ed, Academic Press, Vol 1, pp 412-452), using 1 μ g plasmid DNA for electroporation of 10^7 cells.

Plasmids JV158 and JV10PSA were transformed to *Dictyostelium*, as well as a MB12n control plasmid. After electroporation, the cells from one cuvette were seeded in a 10 cm plate. 12 hours after electroporation blasticidine was added to a final

concentration of 5 µg/ml. 24 hours after electroporation the medium, containing dead cells, was aspirated, the cells were resuspended by pipetting in medium with blasticidine, and the cells were distributed over 24 wells in a 96 well microtiter plate. The 24 wells were each serially diluted 10, 100 and 1000 fold. The medium
5 containing blasticidine was replaced every 3 days. Positive wells were identified 5-9 days after seeding, and transformation efficiency was estimated from the dilution series. Typically, between 1×10^4 and 6×10^4 colonies per µg DNA were obtained. Single wells were then selected for further experiments. A single well contains 200 µl of medium, sufficient for a DELFIA® hLH assay which has a 100% cross-reactivity
10 with hCG. The assay is based on the direct sandwich technique, in which monoclonal antibodies directed against a specific antigenic site on the β-subunit are immobilized. After binding of intact (single chain) hCG to the solid phase antibody, europium-labelled antibodies directed against a specific antigenic site on the α-subunit are bound and quantified. The assay is performed as described by the manufacturer
15 (Wallac Oy, Turku, Finland).

The results clearly show that immunological active single chain hCG is produced from both expression constructs and not from the control plasmid. Moreover, the amount of single chain hCG produced from JV158 (267 mU/ml) seems to be considerably higher than the amount produced from JV10PSA (4.9 mU/ml),
20 suggesting that in this case the human β-hCG leader peptide is more effective than the *Dictyostelium* leader peptide from PsA.

The kinetics of single chain hCG production were studied for JV158 transformed cells. Different densities of transformed cells were seeded on plates in axenic media,
25 grown to confluence and maintained for several days, without any medium change. An aliquot of medium was taken every 24 hrs and analyzed for the presence of single chain hCG. The expression-level reaches a maximum 4 to 5 days after cells reach confluence (data not shown). Since the cells start to detach from the plate at about 6-7 days after reaching confluence, medium was harvested at day 5 after reaching
30 confluence.

Example 3:*Activity of single chain hCG expressed in Dictyostelium*

The ability of single chain hCG from *Dictyostelium* to bind to the human LH/CG receptor was determined by a competitive binding assay with heterodimer hCG. CHO cells expressing the human LH/CG receptor (Jia, X.-C. et al. (1991) Mol. Endocrinol. 5, 759-768) were simultaneously incubated with ¹²⁵I-hCG and purified material from the cell culture supernatant of *Dictyostelium*.

The receptor-binding activity of purified single chain hCG was quantified with a radioligand receptor displacement assay on membrane fractions isolated from exponentially growing cells. In a total volume of 0.5 ml buffer (final composition 10 mM Tris-HCl, 5 mM MgCl₂, 0.1% bovine serum albumin, pH 7.4) a fixed amount of membrane protein was incubated with ¹²⁵I-hCG (20,000 cpm, approximately 12 pM) and increasing amounts of competitor protein for 18 hours at ambient temperature. ¹²⁵I-labelled hCG (NEX-106) was obtained from Du Pont de Nemours. Specific binding was 10-12% of the total radioactivity added. After incubation bound and free hormone were separated by centrifugation. Highly purified, recombinant gonadotropins were used as standards.

For purification of hCG from *Dictyostelium* cell culture medium was harvested from large (22 x 22cm) culture plates. Purification was performed with the aid of a programmable FPLC system (Pharmacia, Roosendaal, The Netherlands) using the control and chromatography supervision system UNICORN (Pharmacia, Roosendaal, The Netherlands). Purification of single chain hCG was accomplished using a combination of hydrophobic interaction and immuno chromatography with LH/CG β-subunit specific monoclonal antibodies. For this purpose, 150 mls of medium were produced with a single chain hCG content of 0.372 unit/ml as determined by DELFIA®. Using this medium, approximately 3 units of purified single chain hCG (~5% yield) were obtained. All procedures were carried out 4°C.

Coincubation with varying concentrations of wildtype hCG, wildtype hLH or single chains hCG displaced ¹²⁵I-hCG binding in a dose-dependent manner (Fig. 2). The displacement curves indicate the material produced by *Dictyostelium* is indeed able to bind to the human LH/CG receptor. The affinity for the receptor is comparable to the affinity of single chain hCG produced by CHO cells, which has been shown to be considerably less effective in displacing ¹²⁵I-hCG binding than the heterodimer of

hCG.¹ Thus, there seems to be no significant difference in binding capacity between single chain hCG produced in *Dictyostelium* compared to CHO cells.

5 The bioactivity of single chain hCG from *Dictyostelium* was analyzed by examination of its ability to stimulate cAMP production in CHO cells expressing the human LH/CG receptor. Cells were incubated for 4 hours with increasing concentrations of hormone in the presence of 0.1 mM 3-isobutyl-1-methylxanthin. The extracellular cAMP was determined by RIA (Immunotech).

10 The results demonstrate that the single chain hCG produced by *Dictyostelium* is indeed able to activate the human LH/CG receptor which results in the production of cAMP. Only a small difference in potency was observed between single chain hCG produced in *Dictyostelium* compared to the material produced in CHO cells (IC₅₀ value approximately five-fold lower).

15 **Example 4:**

Expression and bioactivity of heterodimeric hCG in Dictyostelium

We aimed for the expression of heterodimeric hCG in *Dictyostelium*. For the expression of the α - and β -subunits of hCG, two plasmids were generated. Their structure and overall organisation is essentially identical to that of MB12n which has
20 been described in example 1. In order to facilitate expression of two independent plasmids in Dd we replaced the blasticidine resistance cassette in MB12n with the 2.4 kb KpnI-XbaI fragment from p155d1 (Hughes, J.E. et al. (1994) Mol. Cell. Biol., 14, 6117-6124) containing a neomycin cassette, creating MB12neo. For the construction of the expression vector for the α -subunit, its natural cDNA sequence was produced by
25 PCR using primers which introduce a BglII restriction site both at the 5' and 3' end of the fragment, so that it could be cloned in MB12neo. For the construction of the expression vector for the β -subunit of hCG, MB12n was modified to contain another unique restriction site (SphI) 3' in the BglII cloning site [see example 1]. The hCG β -subunit cDNA was amplified using a 5' primer resulting in the alteration of the first 30
30 bases of the coding sequence conform the *Dictyostelium* preferred codon usage [see example 1]. The primers also introduced appropriate restriction sites at both the 5' (BglII) and 3' (SphI) end of the fragment to facilitate directional cloning.

The two expression plasmids were transformed simultaneously to *Dictyostelium*. After transformation, cells were plated to clonal dilution. Clonal transformants were then selected and further grown for analysis. The amount of hCG secreted by *Dictyostelium* was determined as described by the manufacturer using a DELFIA® hLH assay (Wallac Oy, Turku, Finland), which has a 100% cross-reactivity with hCG. The results clearly show that immunologically active hCG is indeed produced by *Dictyostelium*.

Although the presence of heterodimeric hCG was demonstrated in the medium by means of epitope detection, additional experiments were necessary to establish whether hCG produced by *Dictyostelium* is biologically active. Quantification was based on immuno-reactivity. The bioactivity of heterodimeric hCG from *Dictyostelium* was analysed by examination of its ability to activate the human LH/CG receptor in a luciferase reporter assay. The results demonstrate that the heterodimeric hCG produced by *Dictyostelium* is indeed able to activate the human LH/CG receptor (Figure 4). Moreover, its bioactivity is comparable (IC_{50} value approximately two-fold higher) to the bioactivity of wildtype hCG produced by CHO cells.

Example 5:

Expression and bioactivity of heterodimeric FSH in Dictyostelium

To investigate if *Dictyostelium* is also capable to produce other complex glycoprotein hormones, we studied the production of FSH in this organism. In line with the strategy for hCG (example 4), two expression plasmids were generated. For the construction of the expression vector for the β -subunit of FSH, the cDNA was amplified using a 5' primer resulting in the alteration of the first 27 bases of the coding sequence conform the *Dictyostelium* preferred codon usage and cloning was performed as described for the β -subunit of hCG. After transformation of *Dictyostelium*, cells were plated to clonal dilution. Clonal transformants were then selected and further grown for analysis.

The amount of FSH secreted by *Dictyostelium* was determined by a sandwich immunoassay [SS-artikel]. The results demonstrate that immunological active FSH is produced by *Dictyostelium*. Moreover, heterodimeric FSH produced by *Dictyostelium* is also able to activate the human FSH receptor (Figure 5).

Example 6:*Random mutagenesis of a selected region of hCG in Dictyostelium*

Since we showed that *Dictyostelium* is capable of producing biologically active gonadotropins, we aimed for the development of a random mutagenesis approach. For this purpose, we selected two amino acids in the determinant loop of hCG which have been demonstrated to be involved in receptor binding and signal transduction.

Specific base substitutions were introduced by site-specific mutagenesis and combining PCR-fragments that overlap in sequences as described using standard PCR conditions. The primers were designed to alter aminoacids 94 and 95 of the β -subunit of hCG. The first two nucleotides of both codons were altered fully random (A,C,T or G), while the third base was restricted to G or T to minimise the percentage of stopcodons introduced. The PCR fragments were separated and subcloned in pCR[®] 2.1 (Invitrogen, Leek, The Netherlands). The primers were chosen in such a way that the subcloned PCR-fragments contained a BglII site at the 5' end and a SphI site at the 3' end. After PCR and cloning, the pool of pCR[®] 2.1 constructs was transformed to *E.coli*. Subsequently, DNA was isolated from a pool of 200 transformants and after restriction digestion the BglII/SphI mutated fragments were subcloned in MB12n containing the BglII and SphI site. After plating *E.coli* transformants of MB12 plasmids containing the random mutant fragments, 400 colonies were pooled and DNA was prepared.

Dictyostelium was transformed simultaneously with the expression vector for the α -subunit by electroporation. Selection with blasticidine (10 μ g/ml) was introduced 5 hours after electroporation. The next day, cells were clonally diluted in 96 well plates using 4 fold dilutions and neomycin selection (10 μ g/ml) was initiated. Medium was replaced every 3-4 days, maintaining selective conditions. Positive wells were identified 11-14 days after electroporation, and the transformation efficiency was estimated from the dilution series. Typically, about 500 transformants were obtained by electroporation of 10^7 cells with 1 μ g of both the hCG α and hCG β vectors. Single wells were then selected for further experiments. A single well contains 200 μ l of medium. Larger amounts of media for purification were harvested from large (22 x 22cm) culture plates.

The supernatants of 85 *Dictyostelium* clones were analysed for the presence of immuno- and bioactivity. As controls, several wildtype hCG producing clones and non-transformed *Dictyostelium* clones were present on the 96-well plates. Concentrations of

wildtype and mutant hCG were measured using a DELFIA® hLH assay (Wallac Oy, Turku, Finland), which has a 100% cross-reactivity with hCG. Subsequently, the *in vitro* biological activity on the human LH/CG receptor was determined. Half of the 85 mutants analysed, show B/I ratios varying from 0.35 to 1.05. Taking variations of 2 single analyses into account, the activities of these mutants are considered to be comparable to wildtype hCG. Approximately 40% of the mutants show decreased B/I ratio's. This could be anticipated, since the mutated aminoacids 94 and 95 have been shown to be involved in receptor binding and activation. The fact that 11 clones do not show any hCG production is probably due to interference of the altered aminoacids with appropriate folding of the mutated β -polypeptide and/or association with the α -subunit or the fact that not all clones contain both an α - and a β -subunit expression construct. Twenty clones with varying B/I ratios were selected for detailed analysis.

Selected clones were further grown on 6-well plates until confluence and cell culture supernatants were collected after 4 additional days. Four of the twenty clones did not produce any detectable hCG. This is in agreement with the results for these clones in the initial screen. Among the other clones, the amount of hCG produced varied considerably (116-2118 mU/ml). The cell culture supernatants were analysed for the presence of *in vitro* biological activity of hCG at a range of concentrations. All mutants which showed B/I ratios in the range of wildtype hCG in the initial screen, also displayed a wildtype *in vitro* biological activity when analysed in more detail. The four supernatants that did not show any immunological activity, were also not able to activate the LH/CG receptor. Therefore, we conclude that these clones are not producing any hCG. Furthermore, all mutants with decreased B/I ratios in the initial screen indeed show a clear increase in IC_{50} . Their B/I ratios vary from a 2 fold to >100 fold lower than wildtype hCG produced by *Dictyostelium*. The dose/response curves for eight of the mutants are shown in Figure 6.

To correlate the observed biological activities with the mutations present in the β -subunit, the mutated expression vectors were sequenced. We isolated total DNA from selected *Dictyostelium* clones and transformed it to E.coli. For each *Dictyostelium* clone a number of transformants was analysed by colony-PCR for the presence of the α -subunit plasmid or the β -subunit plasmid. Subsequently, DNA was isolated from several transformants containing the β -subunit plasmid and sequence analysis was performed on the mutated region using an automated sequencer (Pharmacia).

Thus, the majority of *Dictyostelium* clones contains only one type of mutation in the hCG β expression vector. These clones produce a single type of mutant. In addition, we conclude that the majority of *Dictyostelium* clones analysed contain unrelated sequences on position 94 and 95 of the β -subunit, suggesting that the mutagenesis has been random indeed.

Example 7:

Mass spectrometric analysis of rec hCG from Dictyostelium discoideum

Recombinant hCG, produced by *Dictyostelium* was isolated from culture supernatant by subsequently hydrophobic interaction chromatography and immunochromatography with MoAb 119A that reacts with an epitope common to LH and hCG. The antibody was immobilised to NHS-Sepharose. The acid-eluted, purified rec hCG was dialysed against 50% acetonitrile and lyophilised. The protein was dissolved in 0.1% TFA and mass spec analysis was carried out with MALDI-TOF using superDHB as matrix. Pure rec hCG produced by CHO cells and isolated with the same purification procedure was used as a reference.

Both α - and β - subunits of the *Dictyostelium* material were of lower molecular weight than the corresponding subunits from CHO cells, the median values of the peaks being 11578 and 17351 D for the *Dictyostelium* subunits and 14013 and 23284 D for the CHO polypeptides. This observation indicates that less and/or shorter carbohydrate chains are present on the *Dictyostelium*-produced hormone. Moreover, the peak width of the corresponding subunits differed considerably i.e. 11110-12452 D (1342 D) for the *Dictyostelium* α - vs 12959-15591 D (2632 D) for the CHO α -chain and 17006-18104 D (1098 D) for the *Dictyostelium* β vs 20674-25208 D (4534 D) for the CHO β -chain. This indicates that the glycosylation of the *Dictyostelium* product is far less complex.

CLAIMS:

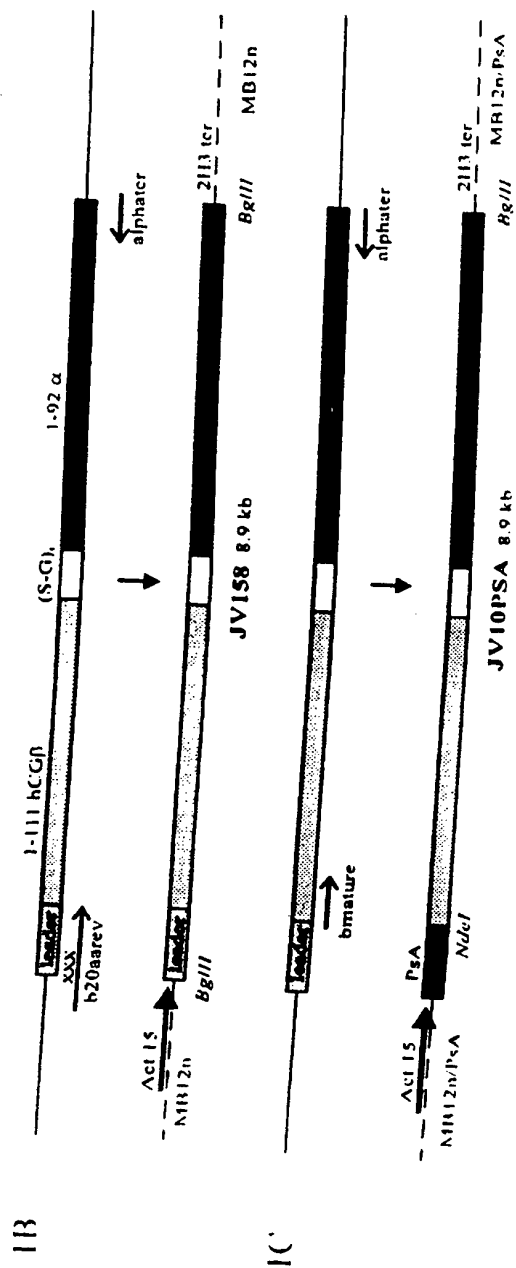
1. A gonadotropin or a mutant thereof obtainable by heterologous expression in a
5 *Dictyostelium* host.
2. The mutant gonadotropin of claim 1 characterized in that the subunits are covalently linked.
3. The gonadotropin according to claims 1 or 2 for use as a therapeutical substance.
4. The gonadotropin according to claims 1-3 characterized in that said gonadotropin is
10 hCG or FSH.
5. A pharmaceutical composition comprising the gonadotropin according to claims 1 or 2 and a pharmaceutical acceptable carrier.
6. A method for producing a gonadotropin or a mutant thereof comprising the steps of:
15
 - transforming a strain of *Dictyostelium* with a recombinant plasmid vector comprising a DNA sequence encoding the gonadotropin genes or mutated genes under control of *Dictyostelium* regulatory sequences
 - culturing the recombinant strain under conditions to allow expression of the DNA sequence and
 - 20 - isolating of the expressed protein.
7. A method for selecting a gonadotropin mutant with super agonistic or antagonistic properties comprising the steps of:
 - random mutagenesis of gonadotropin genes
 - insertion of the mutated genes in a *Dictyostelium* plasmid vector
 - 25 - transforming a strain of *Dictyostelium* with said recombinant plasmid vector
 - culturing clones under conditions to allow expression of the DNA sequence
 - determining the receptor binding / signal transduction ratio of the expressed protein
 - isolating clones with a ratio deviating from the ratio determined for wild type gonadotropins.
 - 30

1/10

1A



Fig. 1



2/10

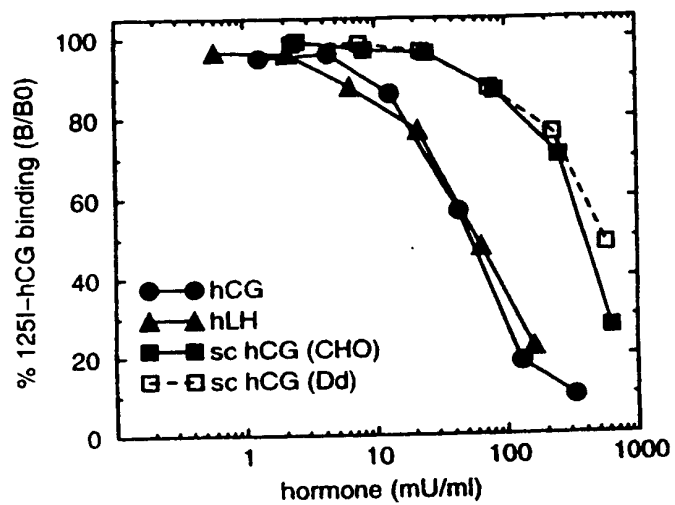


Fig. 2

3/10

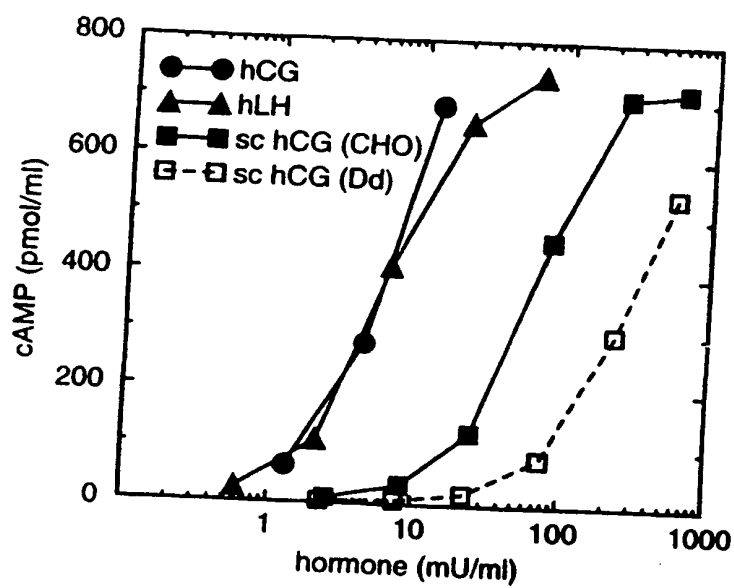


Fig. 3

4/10

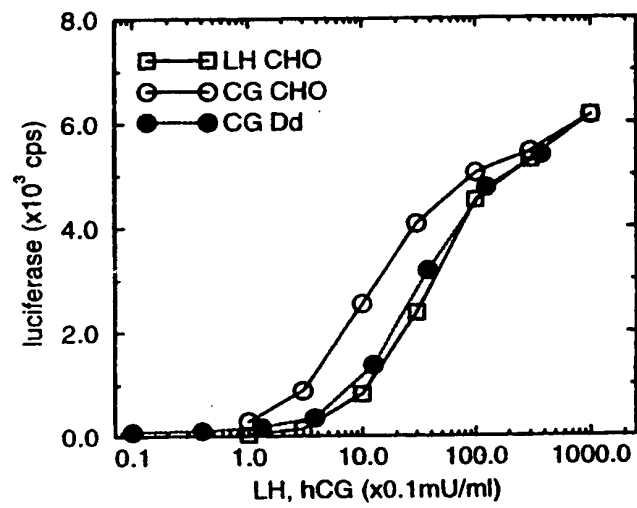


Fig. 4

5/10

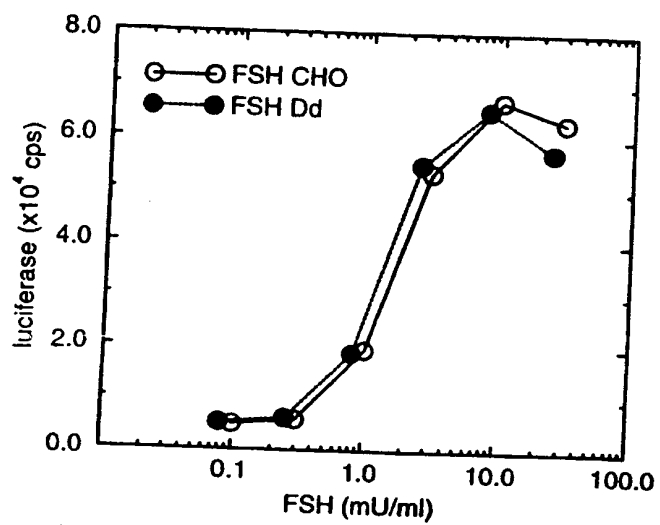


Fig. 5